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Alloxan Cytotoxicity *in vitro*

MICROSCOPE PHOTOMETRIC ANALYSES OF TRYPAN BLUE UPTAKE BY PANCREATIC ISLET CELLS IN SUSPENSION

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Suspensions of islet cells were prepared by shaking pancreatic islets from non-inbred *ob/ob* mice in a Ca^{2+}-free buffer. The cells were incubated with or without 20mM-alloxan, and subsequently with Trypan Blue. The uptake of Trypan Blue by cell nuclei was analysed by microscope photometry and by counting the frequency of cells appearing stained on visual inspection. Cells classified as stained or unstained by inspection showed no overlap in nuclear absorbance. Suspensions not exposed to alloxan contained 70–80% of unstained cells. Alloxan markedly decreased the frequency of unstained cells, an effect counteracted by 5 or 20mM-D-glucose. The spectrum of Trypan Blue in islet-cell nuclei was red-shifted by about 20nm. A similar red-shift was observed on adding the dye to solutions of albumin or histones, but not on mixing the dye with DNA. Binding to basic proteins may explain the concentrative uptake of Trypan Blue in dead cells and contribute to the oncogenic transformation of phagocytotically active cells. β-Cells *in vitro* are killed by alloxan and hence represent a valid model for studying the diabetogenic action of the drug.

As shown in the preceding paper, alloxan inhibits the accumulation of Rb^+ in isolated pancreatic islets or islet cells in suspension (Idahl *et al.*, 1977). Like the diabetogenic action of alloxan *in vivo*, the action of alloxan on Rb^+ pumping can be prevented by D-glucose or 3-*O*-methyl-D-glucose, but not by L-glucose. Blockade of the univalent-cation pump may thus be an essential facet of the mechanism by which alloxan kills the β-cells and induces experimental diabetes mellitus.

Although an irreversible arrest of cation pumping is likely to be incompatible with β-cell survival, the Rb^+-flux data themselves give only an indirect clue to the general viability of the β-cells. To establish a model *in vitro* for studying the diabetogenic toxicity of alloxan, complementary studies of some other parameter are necessary to test whether the glucose-sensitive action of the drug leads to β-cell death *in vitro* as it does *in vivo*. We have therefore investigated the effect of alloxan on the uptake of Trypan Blue by islet cells in suspension. The method was validated by microscope-photometric measurements on a large number of cell nuclei. To gain insight into the mechanism of nuclear dye uptake, the spectrum of Trypan Blue in islet-cell nuclei was compared with those of solutions of Trypan Blue alone or in combination with albumin, histones or DNA.

Materials and Methods

Animals and isolation of islet cells

Male non-inbred *ob/ob* mice (7 months old) from the Umeå colony were starved overnight. Islets containing more than 90% β-cells were obtained by collagenase digestion (Lernmark, 1974) of the pancreatic glands in tissue-culture medium 199 (Salk *et al.*, 1954) containing 5mM-D-glucose and modified to contain 20mM-Hepes [2-(*N*-2-hydroxyethylpiperazin-*N'*-yl)ethanesulphonic acid]. The number of islets isolated for each experiment ranged from 37 to 130.

The islets were transferred to 200μl of tissue-culture medium containing glucose and Hepes as above but modified to be Ca^{2+}-free by the exclusion of calcium salt and the inclusion of 1mM-EGTA. By shaking for 10s at room temperature (about 22°C), the islets were broken up to a milky suspension of cells (Lernmark, 1974). To separate intact cells and cellular debris, the suspension was layered on top of 10ml of 4% (w/v) bovine serum albumin in tissue-culture medium (2.6mM-Ca^{2+}, 5mM-D-glucose, 20mM-Hepes); the dense albumin solution was kept in a centrifuge tube and formed a column about 8cm high. After centrifuging for 5min at 50–70*g*, the pelleted cells were resuspended in 200μl of tissue-culture medium containing 2.6mM-Ca^{2+}, 5mM-D-glucose and 20mM-Hepes, and further supplemented with 1% (w/v) bovine serum albumin. The same medium was used as basal incubation medium in subsequent incubations.

Incubations with alloxan

Cell suspension (50μl) was added to 1ml of basal medium and incubated for 15min at 37°C. From a freshly prepared stock solution of 2M-alloxan in

10mM-HCl, 10μl was gently mixed with the incubated cells by turning the incubation tube upside-down once. Control cells were mixed with 10μl of 10mM-HCl without alloxan; in some experiments the alloxan had been kept at pH12 for 2 days to decrease its diabetogenic activity (alkali-treated alloxan). After continued incubation at 37°C for 15min, the tubes were centrifuged for 2min at 50*g*. The supernatant was removed to leave the cells in about 50μl of medium, which was mixed with 50μl of a solution of Trypan Blue in Hepes-buffered tissue-culture medium; before being added to the cells, the dye solution was passed through a Millipore filter.

Analyses of the Trypan Blue uptake by islet cells

Samples of the stained islet-cell suspensions were taken for microscopic analyses 5, 25, 60 and 85min after the addition of Trypan Blue. A drop of the suspension was placed between object and cover glasses. The frequency of cells with Trypan Blue-stained nuclei was estimated by inspecting and counting 100–500 randomly selected cells, by using a 40× magnification dry lens. The counting of each specimen was completed in 10min.

The validity of the counting procedure requires that the distinction between stained and unstained cell nuclei is free from subjective bias. A special series of experiments was performed to test whether this is the case. Islet cells were treated with alloxan or only HCl as above. About 5min after the addition of Trypan Blue, a mount of alloxan-treated cells was examined in a Zeiss microscope photometer. Each cell was first classified as being stained or unstained by mere inspection through the ordinary eyepieces. The absorbance of the nucleus was then measured at 610nm. After measuring ten stained nuclei, ten unstained nuclei were selected and measured in the same cell mount. This mount was then replaced by one of control cells, among which ten stained and ten unstained nuclei were similarly measured. The procedure was repeated with such alternating sequences of measurements on alloxan-treated and control cells until 30 stained and 30 unstained nuclei had been measured in each group. The whole measuring procedure took 2–3h in each experiment.

Absorbance spectra of stained nuclei and dye-containing medium were taken with the microscope photometer on alloxan-treated cells. Spectra of Trypan Blue were also taken with a Zeiss PMQ II or a Turner 210 spectrophotometer. The Turner instrument was used for recording the interaction of dye with human serum albumin, histones, DNA or salmon sperm nuclei in aqueous solutions.

Chemicals

Alloxan monohydrate was from Nutritional Biochemicals Corp., Cleveland, OH, U.S.A.; Trypan Blue was from ChromaGesellschaft Schmid and Co., Stuttgart-Untertürkheim, Germany; collagenase was from Worthington Biochemical Corp., Freehold, NJ, U.S.A.; tissue-culture medium with or without calcium was from the National Bacteriological Laboratories, Stockholm, Sweden; human serum albumin was from Kabi AB, Stockholm, Sweden; bovine serum albumin (fraction V), calf thymus histone types II-A and VI, calf thymus DNA type I and crude salmon sperm nuclei type II-S were from Sigma Chemical Co., St. Louis, MO, U.S.A. Other chemicals were of analytical grade.

Results

Spectra of Trypan Blue in aqueous solutions and in islet-cell nuclei

Trypan Blue-stained nuclei of islet cells exhibited maximum light-absorption around 610nm (Fig. 1). The spectrum was red-shifted in comparison with that of Trypan Blue alone in aqueous solution, which showed a peak absorbance around 590nm. The

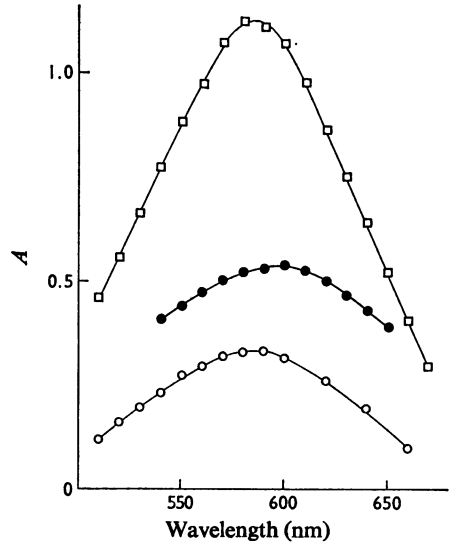

Fig. 1. *Absorbance spectra of Trypan Blue in islet-cell nuclei and basal medium*

Cell nuclei were analysed in the microscope photometer (●), and Trypan Blue in solution was analysed both in the microscope photometer (○, 2mm light-path) and in an ordinary Zeiss PMQII spectrophotometer (□, 10mm-light-path). The dye was added to the media from a stock made by mixing 50mg of Trypan Blue with 10ml of basal medium and filtering. In comparison with the concentration of the stock after filtering (100%), the concentration of Trypan Blue was 50% in the medium used for staining cells and 1.0% (○) or 0.7% (□) in the media used for taking the spectrum of the dye itself.

spectrum of stained islet-cell nuclei resembled that of Trypan Blue added to solutions of albumin or histone, in all of which cases the spectrum was red-shifted by about 20nm (Fig. 2). A clear red-shift was also observed on mixing Trypan Blue with crude sperm nuclei, but the suspension was fairly turbid and less well suited for spectral analyses (results not shown). Unlike albumin, histone and sperm nuclei, DNA in solution did not react with Trypan Blue to give a complex with red-shifted spectrum (Fig. 2).

Distribution of absorbances in islet-cell nuclei first classified as stained or unstained by visual inspection

Fig. 3 shows the frequency distribution of A_{610} in the nuclei of islet cells first diagnosed as stained or unstained by visual inspection. Whether or not the cell suspensions had been incubated with alloxan, the nuclei of subjectively unstained cells had a mean absorbance that was almost identical with that of the surrounding dye-containing medium. However, on an average, the nuclei of unstained control cells were significantly less dense in colour than the surrounding medium ($P<0.005$), suggesting that Trypan Blue was excluded from the cells.

Cells classified as stained had a higher nuclear absorbance than those classified as unstained. The distribution of absorbances showed no overlap between stained and unstained cells. To evaluate the theoretical chance of overlap, each histogram in Fig. 3 was regarded as representing samples from two normally distributed populations of absorbances. From the estimated standard deviations it was calculated that, for control cells, less than 0.5% of the nuclei in the population with high mean value had absorbances lower than 0.100 and less than 0.05% of the nuclei in the population with low mean value had absorbances higher than 0.100.

The resolution of the absorbances into two distinct groups remained, and even appeared improved, after treatment with alloxan. After alloxan, as few as 0.001% of the nuclei belonging to the theoretical population with high mean value had absorbances

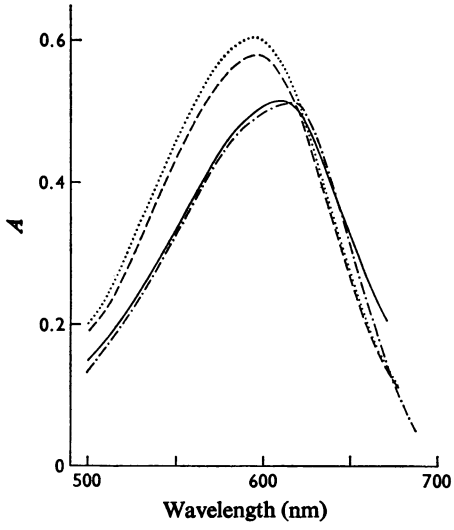

Fig. 2. *Absorbance spectra of Trypan Blue alone* (----) *or in combination with* 3.0*mg of human serum albumin/ml* (-·-·), 2.6*mg of histone type II/ml* (——) *or* 0.25*mg of calf thymus DNA/ml* (· · · ·)

All solutions were buffered with 45–50mM-Hepes (pH7.4) and contained 10μM-Trypan Blue. DNA was added to the buffer after being dissolved in a small volume of 1M-NH_3. The pH of the final solutions was re-adjusted to 7.4 when necessary. The absorbance of albumin, histone type II or DNA in the absence of Trypan Blue was negligible. Histone type VI (0.8mg/ml) gave the same result as shown for histone type II, and salmon sperm nuclei (1.0mg/ml) also produced a similar red-shift; in these cases the absorbance in the absence of Trypan Blue was not negligible, however (not shown). The dye was added from an unfiltered stock of 5.2mM-Trypan Blue (5.0mg/ml) in Hepes buffer. The apparent difference in molar absorbance between Figs. 1 and 2 is due to a marked adsorption of the dye when filtering the stock in Fig. 1.

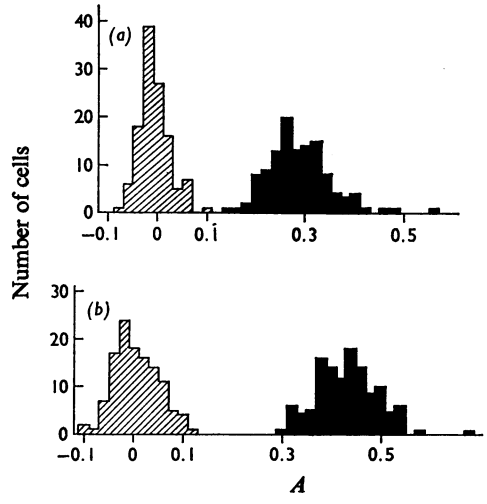

Fig. 3. *Distribution of* A_{610} *in the nuclei of islet cells exposed to Trypan Blue and diagnosed as stained (solid bars) or unstained (hatched bars) by visual inspection*

(*a*) Results with control cells not treated with alloxan; (*b*) results with cells treated with alloxan as described in the Materials and Methods section. Note that the cells were diagnosed as stained or unstained before photometry, and that 120 cells of each type were selected for measurements; the frequency of cells being unstained after various treatments with alloxan is shown in Fig. 4.

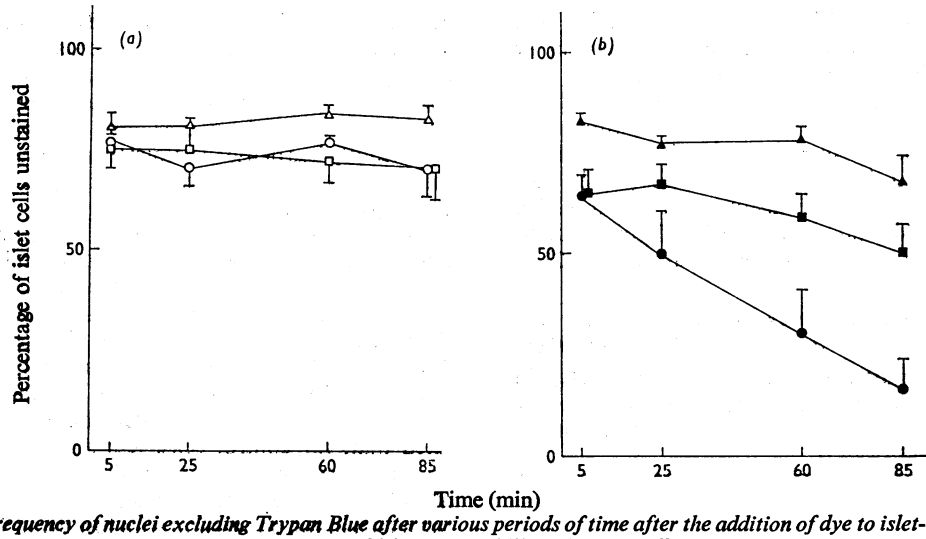

Fig. 4. *Frequency of nuclei excluding Trypan Blue after various periods of time after the addition of dye to islet-cell suspensions not treated (a) or treated (b) with 20 mM-alloxan*

The concentration of D-glucose was 0 mM (○, ●), 5 mM (□, ■) or 20 mM (△, ▲). Results are mean values ± S.E.M. of five different experiments.

Table 1. *Statistical evaluation of the effect of alloxan on the frequency of unstained islet cells*

In each experiment summarized in Fig. 4, the difference between control and alloxan-treated cells was computed at all glucose concentrations and time-points. The means ± S.E.M. as well as the range of these differences are given, together with the probability of alloxan having zero effect. NS, Not significant ($P > 0.05$).

Glucose concn. (mM)	Time (min)	Percentage of unstained cells in controls minus same after alloxan: Mean ± S.E.M.	Range	*P* value: *t* test	Rank sum test
0	5	12.80 ± 4.04	5–27	<0.05	=0.05
	25	21.60 ± 7.72	3–47	<0.05	=0.05
	60	46.60 ± 11.10	18–67	<0.02	=0.05
	85	53.60 ± 6.77	36–74	<0.005	=0.05
5	5	10.40 ± 3.12	4–22	<0.05	=0.05
	25	7.40 ± 2.96	−4–12	NS	NS
	60	13.00 ± 1.67	10–19	<0.005	=0.05
	85	20.20 ± 8.03	0–44	NS	NS
20	5	−0.60 ± 4.07	−12–11	NS	NS
	25	2.80 ± 1.77	3–8	NS	NS
	60	5.40 ± 3.85	−4–19	NS	NS
	85	14.60 ± 4.18	2–23	<0.05	=0.05

lower than 0.100; in the population with low mean value the estimated frequency of absorbances exceeding 0.100 was 1.7%. The improved resolution is due to the fact that alloxan significantly increased the absorbance of nuclei classified as stained with Trypan Blue ($P < 0.001$).

Effect of alloxan on the frequency of Trypan Blue-stained islet cells

The actual percentages of unstained cells in control and alloxan-treated suspensions are summarized in Fig. 4. Table 1 gives a statistical analysis of the effect of alloxan. The frequency of unstained

control cells was about 70–80%, irrespective of the D-glucose concentration and of the time elapsing between the addition of dye and the commencement of cell counting. In the absence of glucose, incubation with 20mM-alloxan significantly decreased the frequency of unstained nuclei at all time-points studied. The effect was time-dependent, since the frequency of unstained nuclei kept falling for more than 1h after exposure to alloxan and dye. In the presence of 5mM-D-glucose, there was a significant effect of alloxan 5min after the addition of Trypan Blue. However, 5mM-D-glucose provided partial protection against alloxan, as indicated by the less dramatic increase of the effect with time. In combination with 20mM-D-glucose, alloxan had no significant effect until 85min after the addition of dye; at that time-point the effect was much smaller than that observed in glucose-free medium.

In six experiments with alkali-treated alloxan the percentage of unstained cells was unaffected 5min (controls: 76.0±1.6; tests: 75.3±2.6) or 25min (controls: 79.2±2.1; tests: 74.2±3.2) after adding Trypan Blue. An effect observed after 60min (controls: 78.3±3.3; tests: 52.3±5.9) and 85min (controls: 77.8±2.0; tests: 60.8±5.5) was clearly smaller than that induced by fresh alloxan.

Discussion

Staining with Trypan Blue has been widely used to assess the viability of eukaryotic cells (e.g. Tennant, 1964; O'Brien & Gottlieb-Rosenkrantz, 1970; Medzihradsky & Marks, 1975). The method is often referred to as the Trypan Blue-exclusion test. However, since the absorbance of stained nuclei is much higher than that of the extracellular dye-containing medium, the stained cells not only fail to exclude Trypan Blue but actually exhibit a concentrative uptake of the dye. As this concentrative uptake is typical of dying or dead cells, it must reflect the physical affinity of the dye for some nuclear component rather than the activity of a transport mechanism in the nuclear envelope. The preferential staining of non-viable cells could be due to a breakdown of diffusion barriers in the plasma and nuclear membranes, to a molecular rearrangement of the chromatin, or to a combination of such factors. That at least one of these membranes is normally impermeable to Trypan Blue is suggested by our observation of a higher absorbance in the surrounding medium that in the nuclei of unstained control cells.

The staining of islet cell nuclei was associated with a red-shift of the absorbance spectrum of Trypan Blue. Commercially available Trypan Blue is known to contain small impurities of azo dyes (Lloyd & Field, 1970; Dijkstra, 1972). The spectral shift in islet-cell nuclei cannot be due to a selective uptake of these impurities, since their absorbance spectra are blue-shifted in comparison with pure Trypan Blue (Dijkstra, 1972). The red-shift in islet cells most likely reflects the binding of Trypan Blue to a nucleoplasmic component, resulting in a dye complex with lower-energy electrons. A similar red-shift has been observed on binding of the dye to plasma proteins (Brenner, 1952; Gestewitz & Wulf, 1973; Wulf, 1973), an effect confirmed here with human serum albumin. In addition, the present results show that histones, but not DNA, produced the same red-shift of the Trypan Blue-absorbance spectrum. Since Trypan Blue is a weak acid, its affinity for basic proteins may be particularly great. Binding to nuclear histones could therefore explain both the red-shift of the spectrum and the concentrative uptake that makes the nuclei stand out in such contrast with the faintly stained cytoplasm of the same cells. Viable reticuloendothelial cells take up Trypan Blue by phagocytosis, and the dye may be stored in cytoplasmic granules for over a year (Schmidt & Thorn, 1970). These cells are also liable to oncogenic transformation in response to the dye (Brown *et al.*, 1963). Perhaps the transformation follows from the binding of small amounts of Trypan Blue to the nuclear histones, resulting in an adverse activation of the genome.

When using Trypan Blue exclusion as a viability criterion, the frequency of stained nuclei is commonly estimated by subjective inspection. The present microscope-photometric analyses indicate that such subjective inspection is a valid method, at least when applied to islet cells. The overlap in absorbance was very small between cells first classified as stained or unstained according to the visual impression. Against this background of objective validation of the technique, it can safely be inferred that alloxan increases the frequency of Trypan Blue-stained islet-cell nuclei. Since staining with Trypan Blue is among the least sensitive indices of functional cell damage (Yuhas *et al.*, 1974; Medzihradsky & Marks, 1975), the marked increase in stained nuclei most probably reflects the same severe β-cell damage as that underlying the induction of experimental diabetes *in vivo*. This conclusion is reinforced by the finding that, like the diabetogenic action and the inhibition of Rb^+ pumping *in vitro* (Idahl *et al.*, 1977), the increased staining with Trypan Blue is counteracted by performing the alloxan treatment in the presence of D-glucose. Hence isolated islets or islet cells represent a valid model for studying the diabetogenic action of alloxan.

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